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(54) Title: <b>POLYMORPHISM AT CODON 36 OF THE P53 GENE</b>  (57) Abstract  A polymorphism at codon 36, exon 4, of the p53 gene has been identified. Methods for ascertaining genetic linkage of the polymorphism with germline mutations in p53 which lead to tumor development, for ascertaining parental origin when such linkage is ascertained, and for determining familial inheritance are provided.		

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## POLYMORPHISM AT CODON 36 OF THE P53 GENE

### Field of the Invention

This invention relates to screening methods based upon the identification of a polymorphism at codon 36, in exon 4, of the p53 gene.

### Background of the Invention

The p53 gene on chromosome 17, specifically region 17p, is the most commonly altered gene in human cancer. Rearrangements of the gene resulting in inactivation have been observed in human and animal cell lines and tumors. The wild type gene product is hypothesized to function in tumor suppression. Consequently, there has been considerable interest in detecting mutations in this gene in tumors. A number of studies have already reported mutations in tumors of diverse origin. Most of the mutations which have been detected have been detected in exons 5-9 of the gene. It has also been reported that transfection of the wild-type p53 arrests the growth of cancer cell lines. (Baker, et al., *Science*, 249:912-915 (1990); Friedman, et al., *Proc. Natl. Acad. Sci. USA*, 87:9275-9279 (1990)).

Most of the p53 mutations identified are "missense" mutations resulting in a single amino acid change. In a recent review article, it was reported that 83% of the p53 mutations are such missense mutations. Harris, et al., "Clinical Implications of the p53 Tumor-Suppressor Gene," *The New England Journal of Medicine*, 329:1318-1327 (1993) (incorporated herein by reference).

One problem hindering the identification of mutations in the p53 gene is the occurrence of sequence polymorphisms, some of which are restriction fragment length polymorphisms

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(RFLPs), in this same gene. Comparison with normal tissue from a patient having a tumor exhibiting a purported mutation in p53 must be made to verify whether the mutation is somatic or germline in origin. Another possibility is that the change in the germline is a polymorphism. Silent mutations in the germline can be indistinguishable from a polymorphism, unless recognized as such in larger population based studies.

Analysis of germline tissues from patients is not always feasible, as many diagnoses are made retrospectively. Thus, the identification of polymorphisms in this gene can aid interpretation of results from tumors.

Several polymorphisms have already been identified in p53, but only three others are in the coding sequence--the rest are in introns. A continually updated compendium will aid in diagnostic interpretation. Discussion of the identified polymorphisms follows.

Ahuja, et al., "Variation in the protein coding region of the human p53 gene," *Oncogene*, 5:1409-1410 (1990) report a polymorphism at residue 21 of the p53 gene in the vicinity of exon 2. The polymorphism was only identified in 1 of 106 samples analyzed and was identified using *Bgl*II. The authors report that the rarity of their polymorphism limits its usefulness in studies of allelic loss.

Ara, et al., "Codon 72 polymorphism of the TP53 gene," *Nucleic Acids Research*, vol. 18, no. 16, 4961 (1990) report a polymorphism at residue 72, exon 4, of the p53 gene. The polymorphism results in an amino acid residue alteration and can be identified using *Acc*II. The frequency of the polymorphic allele is reported as 0.64. The frequency of heterozygosity is not reported, nor is it ascertainable from the reference.

In de la Calle, et al., "Biallelic *Bgl* II DNA polymorphism of the human p53 oncogene," *Nucleic Acids Research*, vol. 18, no. 1, p. 206 (1990), the authors report a polymorphism somewhere in nucleotides 1 to 1587. The polymorphism can be identified using *Bgl*II. The frequency of the polymorphic allele is reported as 0.05.

In de la Calle-Martin, et al., "MspI polymorphism of the human p53 gene," *Nucleic Acids Research*, vol. 18, no. 16, 4963 (1990), a polymorphism in the coding region of p53 is reported upon. The polymorphism can be identified using MspI. 5 The frequency is reported as 0.72.

In de la Calle-Martin, et al., "AccII polymorphism of the p53 gene," *Nucleic Acids Research*, vol. 18, no. 16, 4963 (1990), the authors report a polymorphism in exon 4 of p53. The polymorphism can be identified using AccII. The frequency 10 is reported as 0.68.

Chumakov, et al., "BstNI/NciI polymorphism of the human p53 gene (TP53)," *Nucleic Acids Research*, vol. 19, no. 24, 6969 (1991) report a polymorphism within the 6th intron of the human p53 gene that can be revealed using restriction 15 nuclease digestion. One allele is cleaved by BstNI, the other by NciI. Allele frequencies are reported as 0.31 and 0.69, respectively. Prosser, et al., "Biallelic ApaI polymorphism of the human p53 gene (TP53)," *Nucleic Acids Research*, vol. 19, no. 17, 4799 (1991) report a polymorphism in 20 intron 7 of p53. The polymorphism can be identified using ApaI. The expected frequency of heterozygosity is reported as 10.3 %. The authors suggest that the polymorphism can be used to detect loss of a p53 allele in tumor material.

Carbone, et al., "Polymorphism at codon 213 within the 25 p53 gene," *Oncogene*, 6:1691-1692 (1991) report a polymorphism at codon 213, exon 6, of the p53 gene. The polymorphism was detected in 6 cases and results in the loss of a TaqI site. The authors report that the change would be silent. The authors also report that at least two true somatic mutations 30 alter the same restriction site.

Mazars, et al., "Nucleotide sequence polymorphism in a hotspot mutation region of the p53 gene," *Oncogene*, 7:781-782 (1992) also report a polymorphism in codon 213, in the last base. The authors noted that this is a "hotspot" mutation 35 region. The polymorphism does not change an amino acid. The polymorphism was identified using SSCP. The frequency varied depending upon whether the tissue was of normal or tumor

origin. The highest frequency was observed in a breast cancer patient population. As a result of this observation, the authors attempted to determine whether there was any association with familial breast cancer. None was shown.

5 Willems, et al., "PCR detection of a *EglII* polymorphism in intron I of the human p53 gene (TP53)," *Nucleic Acids Research*, vol. 20, no. 5, 1172 (1992) report a polymorphism in intron I of p53. The polymorphism can be identified using *EglII*. The frequency is reported as 0.08.

10 As already noted above, knowledge of all possible polymorphisms in the p53 gene will facilitate identification of mutations. However, distinguishing between polymorphisms and germline mutations requires larger population based studies. Knowledge of a specific single polymorphism can be used to  
15 eliminate a mutation in p53 as a causative factor in a tumor if the purported mutation displays the same pattern as the polymorphism. In addition to this application, knowledge of a specific single polymorphism can facilitate the determination of the genetic linkage of an identified mutation and,  
20 therefore, the tracing of parental origin and familial histories without requiring laborious sequence determination if the mutation is of germline origin. It should be noted, however, that some germline mutations could arise *de novo*. Genetic linkage of presently unidentified genes distinct from  
25 p53 for which loss of heterozygosity has been linked with cancer or other diseases is also facilitated. Additionally, knowledge of a specific single polymorphism can facilitate determination of loss of heterozygosity. This is particularly important due to the occurrence of loss of heterozygosity of  
30 p53 in tumorigenesis. An association between a p53 mutation and loss of heterozygosity on chromosome 17p has been shown in tumors. Harris, et al., *supra*.

Not all polymorphisms are of equal utility in the aforementioned applications. As a mutation leading to tumor  
35 development is most likely to occur in the coding region, a polymorphism in the coding region, i.e. an exon, is preferable. These regions of the gene are most often studied.

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Polymorphisms in the coding region are generally less common as the coding regions of the gene are more stable and less mutable over time. Further, a coding region polymorphism enables screening of both genomic and cDNA. It is also preferable that the polymorphism result in a change in a restriction enzyme site.

For purposes of genetic linkage determinations, it is further preferable that the polymorphism not occur in the same sites where somatic mutations are known to occur most frequently. Four "hot-spots" in p53 frequently altered in human cancers are reported in Nigro, et al., *Nature*, 342:705-708 (1989) (incorporated herein by reference). These hot spots occur in the conserved regions of the gene. Also, it is preferable that somatic mutations not result in the same detectable change as the polymorphism, but this is a possibility.

For determinations of parental origin, it is preferable that the occurrence of the polymorphism is not too frequent. Preferably, the frequency of heterozygosity should be high enough to be informative, but low enough such that it is not often found in both parents. The homozygous state for the polymorphism is very rare.

The present inventor has identified a polymorphism in codon 36 of exon 4 of the p53 gene which meets the foregoing criteria. It involves the change of a *FlnI* restriction site to a *SccI* site. Although the specific codon change was reported upon previously in Chiba, et al., *Oncogene*, 5:1603-1610 (1990), Chiba et al. reported that the change was a silent mutation in non-small cell lung cancer, not a polymorphism. Chiba et al. do not disclose that the mutation was confirmed as being somatic, nor do they report testing which would confirm whether or not the observation, if not somatic, was a silent germline mutation or polymorphism. For reasons presented above, whether an observed germline mutation is in fact a mutation or a polymorphism is an important difference. The present inventor discovered that the change can be due to a polymorphism. Although the change is "silent" in terms of amino acid coding,

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the change does result in the change from a *FinI* restriction site to a *BccI* restriction site. This was not recognized previously.

#### 5 Summary of the Invention

In one aspect, the present invention involves a method for determining genetic linkage of presently unidentified genes in a region of chromosome 17 for which loss of heterozygosity has been linked with cancer or other diseases. This involves  
10 mapping of the region of genomic loss with respect to other markers in the region to which the gene of interest can be linked. The polymorphism at codon 36 of p53 is one such marker.

In another aspect, the present invention relates to  
15 a method for determining whether a germline mutation in p53 phenotypically expressed as a tumor in a patient is linked to the polymorphism at codon 36, exon 4, of the p53 gene. The method involves isolation of genomic DNA from the tumor of a patient, amplifying the regions corresponding to the mutation  
20 and exon 4 of p53 if necessary, identifying the polymorphism in the amplified sample, cloning the amplified products containing the mutation and the polymorphism, sequencing the cloned products, and determining whether the mutation and polymorphism occur in the same clone. Such occurrence indicates the  
25 mutation is on the same allele. If not, the mutation is on a different allele. In a further aspect, the present invention involves a method for determining parental origin of an allele carrying a germline mutation in p53. The method involves screening tissue from the parents of a patient  
30 exhibiting a tumor with a germline mutation which has been linked to said polymorphism for identification of the parental source of the polymorphism.

In yet another aspect, the present invention involves a method for determining familial histories and, therefore, the  
35 relation of the polymorphism, if any, to tracking of the mutation and predeliction for tumor development. The method involves screening tissue from relatives of a patient



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possessing an allele carrying the mutation for presence of the polymorphism.

In another aspect, the present invention involves a method for determining loss of heterozygosity.

#### 5 Brief Description of the Drawings

Figure 1 depicts the occurrence of the polymorphism in gangliocytoma/ganglioneuroma, neuroblastoma, and normal specimens.

Figure 2 depicts the localization at codon 36 by sequence  
10 determination.

#### Detailed Description of the Invention

Mutations in p53 have been linked with a variety of cancers. Mutations can be somatic or germline in origin, the distinction being that germline mutations are present in all  
15 cells of the body, tumor or otherwise. Many of the mutations observed in p53 are somatic in origin. However, germline mutations, when identified, can be significant due to the possibility they represent genetic inheritance. Germline mutations, however, can also be *de novo*, i.e. originate during  
20 embryogenesis. Although inherited mutations are much less common, they have been identified in family cancer syndromes such as the Li-Fraumeni syndrome. Li et al., "Rhabdomyosarcoma in children," *J. Natl. Cancer Inst.*, 43:1365-1373 (1969). The potential for inherited predilection to cancer development  
25 increases the significance and desirability of screening methods for rapidly and sensitively determining inheritance.

The present invention relates to the identification of a polymorphism at codon 36 in exon 4 of the p53 gene. Exon  
30 4 is the largest exon of the p53 gene and spans from codons 22-125. Although exon 4 does not contain a mutational "hotspot", others have reported mutations in this region. Caron de Fromental, et al., *Genes Chrom. Cancer*, 4:1-15 (1992). An

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example of a mutational "hotspot" is the region spanning amino acids 130 to 240. A number of point mutations were identified in this region, which led to significant, tumor-promoting changes in phenotype. WO 92/00311, published January 9, 1992.

5 See also Nigro, et al., *supra*.

Polymorphisms provide an opportunity for screening methods upon establishment of linkage with a particular mutation. Although the mutations themselves can be screened, the polymorphism may be a restriction fragment length  
10 polymorphism, resulting in the change in length of fragments cleaved with a particular enzyme, such that the presence of the polymorphism can be readily ascertained without resort to sequencing or PCR. Upon establishment of linkage with a mutation, an RFLP allows for simpler analysis, such as in the  
15 establishment of pedigrees.

Additionally, polymorphisms allow for the linkage determination of genes which have not yet been specifically identified, but have been identified in that a loss of heterozygosity in a region purportedly containing the gene  
20 results in the development of cancer or other disease conditions. Thus, pedigree analysis of unidentified genes is also possible. One method used for screening for mutations is single-strand conformation polymorphism (SSCP) analysis. A point mutation in a small DNA fragment causes a significant  
25 conformational change which is detectable as a difference in mobility in non-denaturing acrylamide gel electrophoresis. A limitation of this method is that it does not distinguish between polymorphisms and mutations. The initial identification of polymorphisms by SSCP, or other methods,  
30 followed by characterization by sequencing, should obviate the need for further sequencing when the same polymorphic patterns are found in other cases. Similarly, knowledge of a single specific polymorphism can be helpful. See, for example, the discussion in Mitsudomi, et al., *supra* regarding the purported  
35 mutations at codon 213. During screening, a polymorphism at codon 213 was noticed. To exclude the polymorphism, side by side comparisons with a control sample using SSCP were

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performed. This pattern comparison revealed that 4 of 13 purported mutations in exon 6 may have been polymorphisms.

Various methods are available in the art for identification of mutations in the p53 gene. For example, see  
5 WO 92/13970, published August 20, 1992; WO 92/00311, discussed previously; WO 93/20233, published October 14, 1993; and EP 0390323, published October 3, 1990 (subject matters of which are incorporated herein by reference). Mutations can be determined using SSCP or denaturing gradient gel  
10 electrophoresis (DGGE) as PCR-based methods. If the mutation changes a restriction site, or results in a large deletion, the mutation can also be initially recognized by Southern blot.

The present invention is not limited to any one particular manner of identifying the mutation. Of course,  
15 mutations can also be identified through the more laborious task of sequencing entire gene isolates or genomic hot spots.

Mutations which have been causatively linked to tumor development and/or progression are preferable. That is, tissues of patients displaying mutations which result in  
20 phenotypic expression of tumors are preferred for subjecting to the methods according to the invention. However, as many mutations lead to a pre-cancerous state and depend upon subsequent mutations in the sister allele for entering the cancerous state, such mutations are also contemplated, as they  
25 provide a mechanism for earlier diagnosis. See discussion in WO 92/00311 discussed, supra. In WO 92/00311, a pre-cancer cell is defined as a cell that has one normal p53 allele and one mutated p53 allele. In a cancer cell, both alleles are mutated. If the present polymorphism is linked to a mutation,  
30 it may be possible to identify this pre-cancer state. However, it is also possible that allelic loss may precede mutation in the sister allele. Such is the case with rhabdomyosarcoma (RMS). Felix, et al., Cancer Research, 52: 2243-2247 (1992) (incorporated herein by reference).

35 Once a mutation has been identified, the method according to the invention can be utilized to determine linkage of the mutation with the polymorphism. Linkage determinations

can provide numerous diagnostic advantages. For example, once linkage is established, allelic loss can also be followed. As discussed above, linkage determinations can also be useful for 17p loci other than p53--distal 17p loci are suspected to be involved in brain tumors. Thus, with the establishment of a linkage, it is then possible to screen for carriers.

Further, once a linkage has been determined, parental origin of the allele carrying the mutation can be ascertained. This could enable back-tracking to determine familial inheritance if the mutation is inherited. As discussed previously, however, not all germline mutations are inherited. Additionally, forward-tracking can be utilized to identify carriers. In this manner, siblings and offspring can be screened for the same polymorphism. In the methods according to the invention, amplification of the DNA region containing the mutation is contemplated if the sample is limited. Amplification can be performed by the technique of polymerase chain reaction (PCR) such as disclosed in U.S. Patent No. 4,683,194 issued to Saiki et al (incorporated herein by reference). Primers directed to the coding sequence of p53, or to a specific exon within the coding sequence, can be utilized. For an example of primers directed to exon 4, see Mitsudomi et al., *Oncogene*, 7:171-180 (1992), incorporated herein by reference. The primers used in Example 1 below were designed by Mitsudomi. The PCR products are then cleaved with restriction enzymes, size separated on agarose gels and the polymorphism identified. The polymorphism can also be identified by SSCP.

Alternatively, the polymorphism can be identified by Southern blot, using restriction enzymes *BccI* and *FinI* run in parallel. Fragment patterns can be compared with a standard containing the polymorphism also subjected to cleavage with the enzymes or with the pattern disclosed herein in Figure 1. DNA isolated from a sample known to contain the polymorphism can be simultaneously subjected to the same amplification and cleavage. If the polymorphism is detected in the sample, genetic linkage can be confirmed by sequencing.

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Upon the establishment of genetic linkage, screening to determine parental origin of the polymorphism provides insight into the potential hereditary origin of the mutation and, hence, the tumor. Parental origin screens can be performed in a manner similar to screening the p53 gene, or region thereof, for possession of the polymorphism. Rather than being performed upon tissue extracted from a tumor, however, such screens are performed upon normal tissue isolated from parents and the patient. The same procedure as described above is then followed. The heterozygous occurrence of this polymorphism is infrequent enough that it is unlikely that both parents would be heterozygous.

A similar procedure as outlined above is performed in order to determine familial carriage of distal related genes, especially when those genes have not been isolated. The presence of the polymorphism in a sibling or offspring might indicate proclivity for the mutation and, hence, tumor development.

#### Materials and Methods

##### 20 Oligonucleotide Primers

The primers used for amplification were obtained from Dr. Mitsudomi. See Mitsudomi et al., *supra*. The primers were derived from intron 3 and intron 4 of p53. The sequences of the primers are as follows:

25           5'AGGACCTGGTCTCTGACTC 3'       intron 3       SEQ ID NO:1  
            3'CATTGAAGTCTCATGGAAGC 5'       intron 4       SEQ ID  
NO:2.

##### Single-strand conformation polymorphism (SSCP)

Point mutations in the p53 gene were detected using a modified version of the PCR/SSCP method (Orita et al., *PNAS USA*, 86:2766-2770 (1989) and *Genomics*, 5:874-879 (1989). Suzuki et al., *Oncogene*, 5:1037-1043 (1990), (incorporated herein by reference. One hundred nanograms of genomic DNA was amplified in a volume of 10  $\mu$ l containing 50 mM potassium

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chloride, 10 mM Tris-HCl (pH 8.3), 1.5 mM magnesium chloride, 0.01% (w/v) gelatin, 1.25 mM each of four dNTPs (Pharmacia), 0.05 µg of the pair of primers, 0.25 units of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) and 0.5 µl of 5 [α-<sup>32</sup>P]dCTP (300Ci/mmol<sup>+</sup>, 10mCi/mmol<sup>+</sup>, Amersham, Arlington, IL, USA). The amplification reaction using a thermal cycler (Perkin-Elmer Cetus) consisted of 94°C for 9 minutes for initial denaturation when using genomic DNA, followed by 35 cycles of 94°C for 1 minute, annealing for 1 minute at 55°C 10 (genomic DNA), extension at 72°C for 2 minutes, and a 7 minute final extension at 72°C. One µl was then cleaved with the enzyme AlwNI in 10 µl to reduce the fragment sizes to those more suitable to SSCP.

Two micro-liters of the digested product was 15 transferred to a 96 well plate; mixed with loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and incubated in a 90°C water bath for 5 minutes to separate the single strands. After heating, the samples were immediately cooled on ice and 2 µl of each sample was loaded 20 onto a 6% acrylamide gel containing 89 mM Tris-borate, 2 mM EDTA, pH 8.3 (1 x TBE). The gel was run at 25 W for 4.5 hours at 26°C using 1 x TBE as running buffer. After electrophoresis, the gel was dried and exposed to Kodak X-Omat AR film with an intensifying screen at -70°C for 15 hours.

## 25 Characterization of Polymorphism

Polymorphisms were confirmed by direct sequencing of genomic DNA/PCR products (Fig. 1). One µg genomic DNA was PCR amplified and 1/250 of these products used as template in a second heminested PCR reaction for sequencing. Thirty cycles 30 at 95°C for 1 minute, 58°C for 1 minute, and 72°C for 3 minutes were utilized. PCR products were agarose gel isolated, and 500 ng were directly sequenced using nested oligonucleotides.

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**Example 1 - Determination of polymorphism**

Exon 4 of the p53 gene was screened by genomic DNA SSCP analysis as describe above on genomic DNA from 100 individuals. Genomic DNAs were prepared from the peripheral blood of 18 normal individuals; from the peripheral blood, 3 lymphoblastoid lines or fibroblast lines, but not tumor tissue, of 16 patients affected with various cancers; 15 leukemic marrow specimens; 20 neuroblastomas or neuroblastoma cell lines; nine rhabdomyosarcomas or rhabdomyosarcoma cell lines; 21 brain tumors; one testicular tumor; and one retinoblastoma cell line. Paired leukemia and brain tumor specimens from one patient with multiple primary cancers were included in the aforementioned samples as reported upon by Perilongo et al., *Leukemia*, 7:912-915 (1993). Matched normal tissues from nine patients with some of the above cancers also were studied.

An identical SSCP pattern difference and silent CCG to CCA change at codon 36 suggesting a p53 polymorphism were found in peripheral bloods of two unrelated normal individuals, matched tumor and normal tissues of a patient with a gangliocytoma/ganglioneuroma, and in a neuroblastoma. The results are depicted in Figure 1. In panel 1, lane 1 is normal peripheral blood, lane 2 is a leukemia cell line, and lanes 3-5 represent brain tumor. In panel 2, lane 1 is a retinoblastoma and lanes 2-9 represent neuroblastomas. In panel 3, lane 1 is a brain tumor, lane 2 is blood from the patient as the sample in lane 1, and lanes 3-5 represent normal tissue samples. T91-84T represents the gangliocytoma/ganglioneuroma specimen. NB4 represents the neuroblastoma specimen. The normal specimen is designated as such.

As can be seen from the figure, four samples out of 100 displayed the polymorphism. All four samples displaying the polymorphism were also heterozygous for the polymorphism. The polymorphism changes a *FinI* restriction site to a *BccI* site. An arrow in Figure 1 represents the pattern of the polymorphic allele. An asterisk indicates the other exon 4 polymorphism at codon 72. The NB4 specimen exhibited both polymorphisms. Other specimens tested were homozygous for the

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more common allele. No specimens tested were homozygous for the polymorphism, tumor or normal.

Genomic DNA from the peripheral blood of a normal individual was amplified by PCR and sequenced directly to  
5 verify the polymorphism and heterozygosity at codon 36. The results are depicted in Figure 2. As can be seen in Figure 2, there are bands present in the "G" and "A" lanes at the 3' terminal nucleotide of codon 36.

#### Example 2 - Determining Genetic Linkage of Unidentified Genes

10 The polymorphism at codon 36 of p53 can serve as a marker for mapping unidentified genes on chromosome 17 for which loss of heterozygosity has been linked with disease. The region of genomic loss can be mapped using standard techniques and the polymorphism. A recent example of how genetic linkage  
15 led to the identification of a new gene is that of the genetic mapping of a locus predisposing to colorectal cancer (Peltomaki et al., *Science*, 260:810-812 (1993), incorporated herein by reference.), where specific chromosomal markers on human chromosome 2 were traced in pedigrees. In this case, the  
20 polymorphisms were microsatellites.

#### Example 3 - Determining Genetic Linkage of Identified p53 mutation

Once a tumor possessing a germline mutation is identified, allelic linkage with the polymorphism at codon 36  
25 of exon 4 can be assessed. Genomic DNA is extracted from the tumor and the presence of the polymorphism pattern can be screened for using Southern blot analysis. The DNA can be cut with *FniI* and *BccI*. The labelled probe to be used can be p53 cDNA, such as the 1.8 kb *XbaI* p53 cDNA probe derived from clone  
30 php53cl discussed in Felix et al., *J. Clin. Invest.*, 89:640-647 (1992) (incorporated herein by reference).

Alternatively, if the sample size so requires, genomic DNA is extracted from the tumor and the region of the gene purportedly possessing the mutation and exon 4 can be amplified  
35 using PCR and the polymorphism screened for using SSCP. The



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particular primers to be used for the PCR amplification will depend upon the location of the mutation in relation to the polymorphism. One skilled in the art could readily determine the appropriate primer to be used from those available in the literature and the sequence of p53 which is available in GenBank. The primers for these purposes would be selected so that the amplified products encompassed both the mutation and the polymorphism. For a list of some primers which can be utilized, see Mitsudomi et al., *supra*.

10 In both instances, the patterns obtained can be compared with genomic DNA from a sample possessing the polymorphism, or the patterns in Figure 1 if testing conditions are the same.

Upon identification of a similar pattern, the amplified PCR product, or genomic DNA region containing the mutation and polymorphism, can be cloned using methods available in the art and the cloned material sequenced. Specifically, in the case of genomic DNA, genomic cloning can be utilized. Briefly, the genomic DNA can be cleaved with restriction enzymes which result in a fragment containing the mutation and the polymorphism. This fragment can then be cloned into a vector with complementary sites. The appropriate restriction enzymes will depend upon the location of the mutation in relation to the polymorphism and can be readily ascertained by one skilled in the art.

25 The presence of the mutation in the same clone as the polymorphism indicates that both occur on the same allele. Conversely, the presence of one and not the other, suggests that they occur on different alleles.

#### 30 Example 4 - Determining Parental Origin

Once allelic linkage has been established between the polymorphism at codon 36 of exon 4 and the germline mutation, genomic DNA can be isolated from tissue from the parents and screened for parental origin of the allele carrying the mutation. The polymorphism can be identified by either Southern blot or SSCP as in Example 3 above.

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**Example 5 - Determining Familial Pedigree**

Once allelic linkage has been established between the polymorphism at codon 36 of exon 4 and the germline mutation, genomic DNA can be isolated from tissue from the siblings, 5 offspring, and other relatives of the patient to screen for the presence of the allele carrying the mutation. The polymorphism can be identified by either Southern blot or SSCP as in Example 3 above.

**Example 6 - Determining Loss of Heterozygosity**

10 Regardless whether linkage between the polymorphism at codon 36 of p53 and the germline mutation is established, the presence or absence of heterozygosity for the polymorphism in the patient sample can be ascertained by the methods discussed above. Specifically, heterozygosity for the 15 polymorphism at codon 36 can be ascertained by SSCP or Southern blotting. In the instance of Southern blotting, both BccI and FlnI would be used. Loss of heterozygosity can be ascertained by comparison with normal tissue from the patient.

The foregoing examples are meant to illustrate the 20 invention and not to limit it in any way. Those skilled in the art will recognize that modifications can be made which are within the spirit and scope of the invention as set forth in the appended claims.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

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5 (ii) TITLE OF INVENTION: Polymorphism at Codon 36 of the p53 Gene

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15 (F) ZIP: 19103

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: PC-DOS

20 (D) SOFTWARE: WORDPERFECT 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: Concurrently herewith

(C) CLASSIFICATION:

25 (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

- 18 -

(A) NAME: Doreen Yanko Trujillo

(B) REGISTRATION NUMBER: 35,719

(C) REFERENCE/DOCKET NUMBER: CH-0486

## (ix)- TELECOMMUNICATION INFORMATION:

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## (2) INFORMATION FOR SEQ ID NO: 1

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

10 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

15 AGGACCTGGT CCTCTGACTC

20

## (2) INFORMATION FOR SEQ ID NO: 2

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: nucleic acid

20 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

CGAAGGTACT CTGAAGTTAC

20

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What is claimed is:

1. A method for determining linkage of a region of chromosome 17 for which loss of heterozygosity leads to development of disease to a polymorphism at codon 36 of exon 5 4 said method comprising:
  - a) isolating genomic DNA corresponding to said region; and
  - b) identifying the polymorphism in said DNA.
2. The method of claim 1 wherein the polymorphism 10 is identified by single-strand conformation polymorphism analysis.
3. The method of claim 1 wherein the polymorphism is identified by Southern blotting.
4. The method of claim 1 wherein the disease is 15 cancer.
5. A method for determining genetic linkage of a previously identified germline mutation in the p53 gene resulting in expression of a tumorigenic phenotype with a polymorphism at codon 36 in exon 4 of said gene in a patient 20 extract, said method comprising:
  - a) isolating genomic DNA from the tumor;
  - b) identifying the polymorphism in said DNA;
  - c) cloning the region of the DNA containing the mutation and the polymorphism;
  - 25 d) sequencing the cloned DNA; and
  - e) ascertaining whether the mutation and polymorphism are present in the same clone.
6. The method of claim 5 wherein the polymorphism is identified using single-strand conformation polymorphism 30 analysis.

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7. The method of claim 5 wherein the polymorphism is identified using Southern blotting.

8. The method of claim 5 wherein the patient has been diagnosed with the Li-Fraumeni syndrome.

5 9. A method for determining parental origin of an allele carrying a germline mutation in the p53 gene which has been linked to a polymorphism at codon 36 of exon 4 comprising:

- 10 a) isolating genomic DNA from each parent of a patient possessing the allele; and
- b) identifying the polymorphism in the DNA.

10. The method of claim 9 wherein the polymorphism is identified using single-strand conformation polymorphism analysis.

15 11. The method of claim 9 wherein the polymorphism is identified using Southern blotting.

12. A method for determining familial inheritance of an allele carrying a germline mutation in p53 which has been linked to a polymorphism at codon 36 in exon 4 of the p53  
20 gene said method comprising:

- a) isolating genomic DNA from relatives of a patient possessing the allele;
- b) identifying the polymorphism in said genomic DNA.

25 13. The method of claim 12 wherein the polymorphism is identified using single-strand conformation polymorphism analysis.

14. The method of claim 13 wherein the polymorphism is identified using Southern blotting.

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15. A method for determining loss of heterozygosity in chromosome 17 in a tumor sample comprising:

- a) isolating genomic DNA from a tumor of a patient exhibiting said tumor;
- 5       b) identifying a polymorphism at codon 36 of exon 4 in said DNA;
- c) determining that the polymorphism is homozygous in said tumor DNA;
- d) isolating genomic DNA from normal tissue of said 10 patient;
- e) identifying the polymorphism in the genomic DNA of said normal tissue; and
- f) determining whether said normal tissue is heterozygous for the polymorphism.

15       16. The method of claim 15 wherein the polymorphism is identified using single-strand conformation polymorphism analysis.

      17. The method of claim 15 wherein the polymorphism is identified using Southern blotting.

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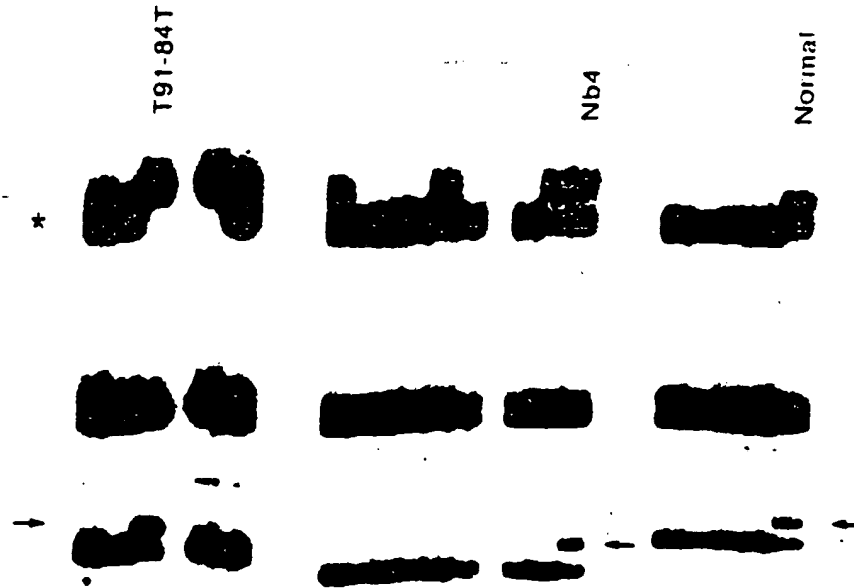


FIG. 1

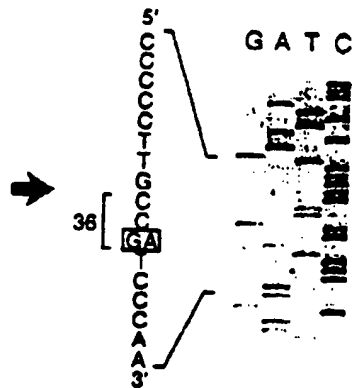


FIG. 2

SUBSTITUTE SHEET



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/00158

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/04; C12Q 1/68

US CL : 536/24.31; 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/24.31; 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CA

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ONCOGENE, Volume 5, issued 1990, Chiba et al., "Mutations in the p53 gene are frequent in primary, resected non-small cell cancer," pages 1603-1610, see especially page 1604, Table 1, sample 831.	1-7, 9-17 -----
Y		8
Y	THE JOURNAL OF CLINICAL INVESTIGATION, Vol. 89, issued February 1992, Felix et al., "Hereditary and Acquired p53 Gene Mutations in Childhood Acute Lymphoblastic Leukemia," pages 640-647, see especially the Abstract.	8

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* "A" Special categories of cited documents documents defining the general state of the art which is not considered to be of particular relevance	* "T" later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principles or theory underlying the invention
* "E" earlier documents published on or after the international filing date	* "X" documents of particular relevance: the cited document cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* "L" documents which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* "Y" documents of particular relevance: the cited document cannot be considered to involve an inventive step when the document is considered with one or more other such documents, such combinations being obvious to a person skilled in the art
* "O" documents referring to an oral disclosure, use, exhibition or other means	* "A" documents member of the same patent family
* "P" documents published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

13 APRIL 1995

Date of mailing of the international search report

28 APR 1995

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